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A Cholesterol-Sensitive Regulator of the Androgen Receptor

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Published studies from our laboratory have shown that critical mediators of tumor cell physiology and behavior reside in cholesterol-rich, lipid raft membranes of prostate cancer cells. This project focuses on the RNA binding protein heterogeneous nuclear ribonucleoprotein K (hnRNP-K) as a novel regulator of the androgen receptor (AR). We have found that hnRNP-K lies within the cholesterol-sensitive PI3K/Akt/PTEN/mTOR pathway and that hnRNP-K connects ErbB receptor/Akt-derived signals with androgenic signals, thereby directly linking peptide hormone and steroid hormone signal transduction mechanisms. Our ***hypothesis*** is that hnRNP-K mediates androgen sensitivity and growth and survival in prostate cancer cells by a mechanism involving the regulation of AR mRNA translation. The specific aims are:

Aim 1. Determine the mechanism(s) underlying the effects of hnRNP-K on androgen sensitivity in prostate cancer cells.
Aim 2. Determine whether changes in expression and/or subcellular localization alter the function of hnRNP-K and assess the physiologic consequences of these changes in prostate cancer cells.

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INTRODUCTION

Published studies from our laboratory have shown that critical mediators of tumor cell physiology and behavior reside in cholesterol-rich, lipid raft membranes of prostate cancer cells. This project focuses on the RNA binding protein heterogeneous nuclear ribonucleoprotein K (hnRNP-K) as a novel regulator of the androgen receptor (AR). We have found that hnRNP-K lies within the cholesterol-sensitive PI3K/Akt/PTEN/mTOR pathway and that hnRNP-K connects ErbB receptor/Akt-derived signals with androgenic signals, thereby directly linking peptide hormone and steroid hormone signal transduction mechanisms. Our ***hypothesis*** is that hnRNP-K mediates androgen sensitivity and growth and survival in prostate cancer cells by a mechanism involving the regulation of AR mRNA translation. The specific aims are:

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BODY

As summarized below, we have made extensive progress on the following tasks:

Aim 1, Task 1. *Determine the mechanism of the effects of hnRNP K on AR expression.*
Aim 1, Task 3. *Generate hnRNP K mutants that differentially regulate the AR.*
Aim 2, Task 1. *Determine the physiological consequences of altering hnRNP K expression in prostate cancer cells.*

HnRNP-K has been shown to be regulate translation of a number of mRNAs and the protein lies downstream from the EGFR in some contexts. Consequently, hnRNP-K is a logical intermediate in a pathway we previously described leading from EGFR to mTOR, in which AR protein levels are down-regulated at the level of mRNA translation [1].

To test the potential involvement of hnRNP-K in this mechanism, we first examined the effect of hnRNP-K on AR expression in LNCaP cells. Enforced expression of hnRNP-K lowered AR protein levels in both the presence and absence of the synthetic androgen, R1881 (1nM), without any effect on the levels of the signaling receptors EGFR and KDR, or on β-actin (Fig. 1A). To determine whether hnRNP-K alters translocation of AR to the nucleus, we evaluated the extent of accumulation of AR in cytoplasmic versus nuclear fractions after enforced expression of hnRNP-K. We found a similar lowering of AR in nuclei and cytosol (Fig. 1A, right panel), indicating that hnRNP-K does not influence AR transit from cytoplasm to the nucleus, while it does alter steady-state levels of AR. Conversely, knockdown of endogenous hnRNP-K by RNA interference (Fig. 1B) increased AR levels, suggesting that endogenous hnRNP-K is conferring the same function as the overexpressed protein. HnRNP-K siRNA also attenuated the suppressive effect of HB-EGF on AR mRNA levels (Fig. 1C), suggesting that hnRNP-K controls AR levels downstream from the EGFR.

In order to assess whether the effect of hnRNP-K on AR is confined to LNCaP cells, (AR-negative) HeLa cells were transiently co-transfected with hnRNP-K along with human AR expression constructs and the effect of enforced expression of hnRNP-K on AR levels was monitored. Our results showed a significant decrease of AR expression in the presence of hnRNP-K in the presence and absence of androgen (not shown), consistent with the findings in the LNCaP cell background.

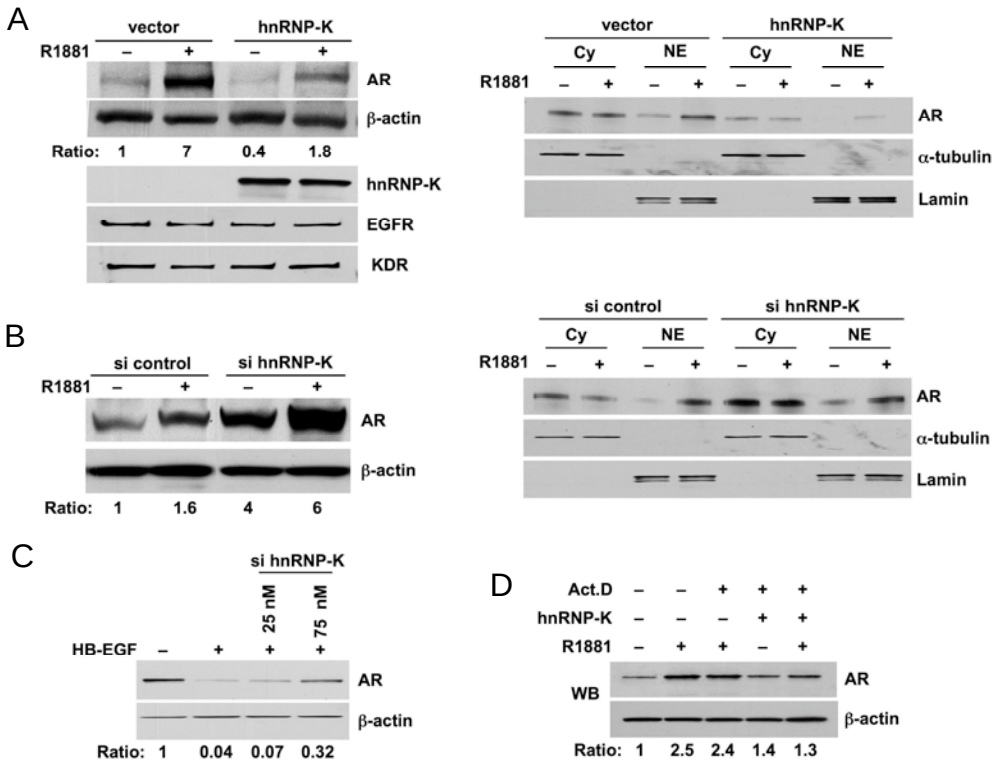

Figure 1. HnRNP-K downregulates AR expression in the presence and absence of androgen. **(A, left panel)** LNCaP cells were transiently transfected with either hnRNP-K or vector constructs using Nucleofection (Amaxa). Protein was transferred to nitrocellulose and blotted either with AR, HA (for hnRNP-K), EGFR, KDR, or β-actin antibody. **(A, right panel)** Fractionation of LNCaP cells before and after hnRNP-K transfection using Lipofectamine 2000 (Cy, cytoplasmic fraction and NE, nuclear fraction); α-tubulin and lamin were used as cytoplasmic and nuclear markers, respectively. **(B, left panel)** Knockdown of hnRNP-K increased AR expression in the presence and absence of 1 nM R1881 (using Nucleofection as transfection method). **(B, right panel)** Nuclear (NE) and cytoplasmic (Cy) fractionation of LNCaP cells after knockdown of endogenous hnRNP-K. **(C)** Knockdown of endogenous hnRNP-K reverts HB-EGF- (EGFR-) mediated down-regulation of AR. **(D)** Steady-state AR protein level in the presence and absence of actinomycin D (5 μM for 24 h) and R1881 (1 nM) after hnRNP-K transfection. In each case, data were quantified using NIH Image 1.63 software and represented as the ratio of AR and β-actin levels.

Post-transcriptional regulation of AR by hnRNP-K. The experiments with HeLa cells suggested that the effect on AR was unlikely to be a result of changes in transcription of AR mRNA. Consistent with this hypothesis, we observed no changes in AR mRNA following enforced expression of hnRNP-K in LNCaP cells (not shown). HnRNP-K over-expression also did not affect AR mRNA levels when cells were treated with the transcription inhibitor, actinomycin D, for up to 20 h (not shown). Actinomycin D similarly did not alter the observed hnRNP-K effects on AR protein levels (Fig. 1D).

The selective mTOR inhibitor, rapamycin, increased AR expression in LNCaP cells, consistent with published results [1] (Fig. 2A). HnRNP-K siRNA evoked a comparable, but not additive, effect on AR protein levels, suggesting that hnRNP-K may act upstream of mTOR (Fig. 2A, right panel). Rapamycin exerts regulatory effects on cap-dependent mRNAs. EGFR activation was previously shown to attenuate AR protein levels in a rapamycin-sensitive manner, at least partly through the AR mRNA 5'-UTR [1]. Thus, we analyzed the effect of enforced expression of hnRNP-K on a 570 nt fragment of the AR 5'-UTR, which contains several cis-acting elements and cap- and stem-loop secondary structures and was shown previously to be rapamycin-sensitive [1]. To do this, we used a luciferase plasmid system in which the 5'-UTR fragment regulates the CMV promoter and that reports effects on cap-dependent mRNA translation. Rapamycin induced luciferase expression from this plasmid construct (Fig. 2B, left panel). In the

presence and absence of rapamycin, transient expression of hnRNP-K in LNCaP cells inhibited luciferase expression. These data strongly suggest that hnRNP-K exerts its attenuating effects on AR expression at the level of mRNA translation. Consistent with this interpretation, we verified the direct binding of hnRNP-K with AR 5`-UTR RNA corresponding to this same UTR region by RNA gel mobility shift in concert with purified GST-hnRNP-K (not shown), and also with endogenous AR mRNA in whole cells using immunoprecipitation/reverse transcription-PCR (Fig. 2B, right panel). The inhibitory effect of hnRNP-K on expression driven by the AR 5`-UTR (Fig. 2B) does not require the AR, since similar findings were obtained in AR-negative COS cells (Fig. 2C, left panel).

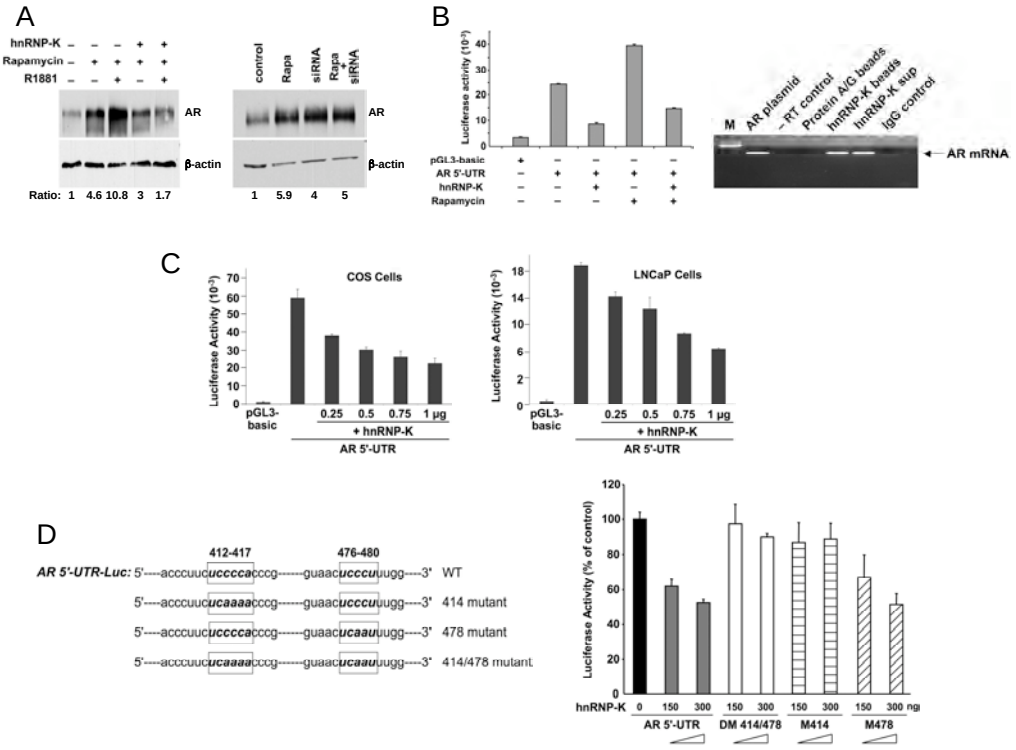

Figure 2. HnRNP-K regulates AR expression at the AR 5'-untranslated region (5'-UTR**). (A, left panel)** LNCaP cells were transfected with hnRNP-K expression plasmid and treated with either 100 nM rapamycin and/or 1 nM R1881. **(A, right panel)** Determination of AR level in presence and absence of hnRNP-K siRNA and 100 nM rapamycin alone or in combination. All data were analysed by NIH Image 1.63 and represented as the ratio of AR and β-actin. **(B, left panel)** Effect of hnRNP-K on AR 5`-UTR luciferase activity in presence and absence of rapamycin. AR 5`-UTR luciferase plasmid (500 ng) or the control plasmid (500 ng) were cotransfected with 500 ng of hnRNP-K in LNCaP cells. Lysates were assayed for luciferase activity and protein content. **(B, right panel)** To verify that hnRNP-K binds to AR mRNA, LNCaP cells were immunoprecipitated with either IgG or hnRNP-K antibody and RNA was extracted and subjected to RT-PCR analysis using AR specific primers. Negative controls included: no added reverse transcriptase (-RT), beads (protein A/G) alone, and IgG immunoprecipitate. Specific PCR band in extracts of LNCaP cells was used as a positive control. **(C)** COS and LNCaP cells were transfected with control plasmid (pIR-Luc) plasmid or plasmid containing the AR 5`-UTR in the presence of varying amounts of hnRNP-K. **(D, left panel)** Schematic representation of AR 5`-UTR-Luc constructs highlighting the conserved hnRNP-K binding motifs (UCCCCA and UCCCU) in human AR mRNA. These two motifs were altered to UCAAAA (414 mutant) and UCAAU (478 mutant). **(D, right panel)** Either AR 5`UTR-Luc wild type, or single (M414 and M478) and double mutants (DM 414/478) were tested for activity in the presence of varying doses of hnRNP-K.

Inspection of the 570 nt rapamycin-sensitive region in the AR 5'-UTR using the consensus sequence U(C)nA/U revealed two potential hnRNP-K binding sites at nt 412-417 (UCCCCA) and 476-480 (UCCCU). To evaluate whether either of these UC-rich regions mediates some or all of the inhibitory effects of hnRNP-K on the translation reporter, we generated single and double mutants within the rapamycin-sensitive fragment (Fig. 2D, left panel). Alteration of the consensus sequence (by mutating UCCCCA to UCAAAA) at the 412-417 site, but not alteration of the 476-480 site, abolished the inhibitory

effects of hnRNP-K on reporter expression (Fig. 2D, right panel), confirming the involvement of the 412-417 site within the AR 5'-UTR in the regulatory effect we observed with hnRNP-K.

HnRNP-K inhibits translation of AR mRNA. HnRNP-K regulates translation of the L2 capsid protein of human papillomavirus type 16 by binding within the open reading frame of the coding region [2]. Inspection of the entire AR mRNA indicated that the message may harbor as many as 9 other potential hnRNP-K binding sites in the coding region and in the 3'-UTR (Table 1) suggesting the possibility that hnRNP-K may also regulate AR expression through one or more of these additional sites. To address this question, we examined the effect of hnRNP-K on translation of AR mRNA using a construct containing only the AR coding region, which contains 6 potential hnRNP-K binding sites (Table 1).

Table 1: Consensus hnRNP-K[U(C)$_n$A/U] binding sites in human AR mRNA

	Position	Sequence
5' UTR	412-17	UCCCCA
	476-80	UCCCU
	664-68	UCCCA
ORF	1403-08	UCCCCA
	1510-14	UCCCA
	1608-12	UCCCU
	2255-66	UCCCCA-UCCCCA
	2656-65	UCCCA-UCCCA
3' UTR	3887-901	UCCCCA
	4090-94	UCCCA
	4115-19	UCCCA

AR was synthesized with a coupled transcription/translation system, using either [^{35}S]methionine for radioactive detection or unlabeled methionine for Western blot identification. Addition of purified GST-hnRNP-K dose-dependently decreased the synthesis of the 100 and 110 kDa translated products (Fig. 3). In contrast, no translation inhibition was observed on the internal control luciferase mRNA (lower panels). Results with cold methionine (Fig. 3B) confirmed the identity of the translation products as AR by immunoblotting. In a complementary experiment, enforced expression of hnRNP-K in HeLa cells reduced the expression of AR expressed from the same hAR construct used in the coupled transcription-translation experiments (not shown). These findings indicate that hnRNP-K employs one or more binding sites within the coding region in a manner that is functionally compatible with the single binding site we identified in the rapamycin-sensitive region of the 5'-UTR.

In an independent test of the ability of hnRNP-K to act as a regulator of AR translation, we probed the distribution of AR mRNA in polysomal fractions isolated from LNCaP cells under conditions where hnRNP-K expression was enforced. If hnRNP-K acts as an inhibitor of AR mRNA translation at the level of *initiation*, then its forced expression would result in the dissociation of AR mRNA from actively translating ribosomes and a re-partitioning of the mRNA toward the lighter fractions of the gradient. As indicated in Figure 3C (left and right panels), hnRNP-K had no such effect on the distribution of AR mRNA in gradient fractions, suggesting that the protein does not inhibit translation initiation of AR mRNA. HnRNP-K had no effect on the distribution of tubulin mRNA (Fig. 3C, bottom panels), as expected. Next, we tested the possibility that the AR mRNA under conditions of enforced hnRNP-K expression was associated with translationally-inactive ribosomes, an event generally associated with a defect in peptide elongation. To test this, we used puromycin, which causes premature termination and release of all actively elongating mRNAs from polysomes. If an mRNA species is not being actively translated, puromycin will not be incorporated into the peptide chain and mRNA release from polysomes will not occur. In the absence of elevated hnRNP-K, puromycin caused a release of tubulin and AR mRNAs from the polysomal fractions (compare Fig. 3C left panel, with 3D left panel, particularly lanes 8-11 which

contain AR and tubulin mRNAs associated with large polysomes). However, when hnRNP-K expression was enforced, AR mRNA was retained in heavy polysomal fractions, even after puromycin treatment (compare Fig. 3C and D), indicating that hnRNP-K evokes a reduction in rate of AR mRNA chain elongation.

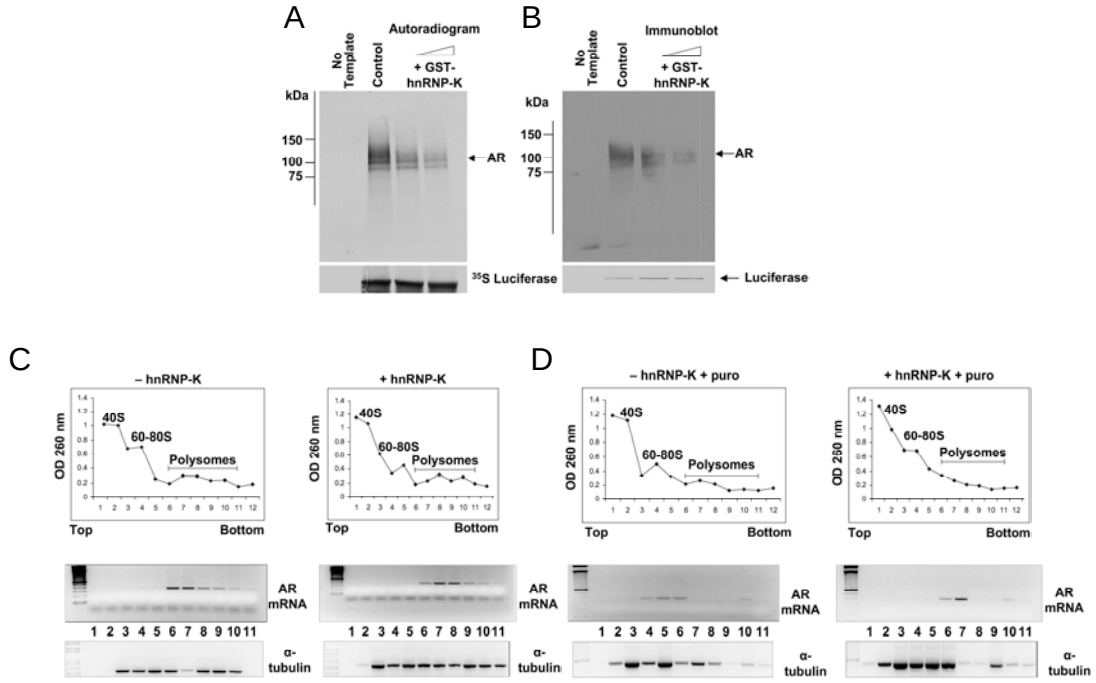

Figure 3. HnRNP-K inhibits the translation of AR mRNA. **(A)** Effect of purified GST-hnRNP-K on translation of AR mRNA as determined by coupled in vitro transcription/translation using [^{35}S]methionine. **(B)** Western blot analysis with monoclonal AR antibody after *in vitro* translation with unlabeled methionine. Luciferase (lower panel) was used as a translation control in A and B. **(C)** LNCaP cells were treated with R1881 in the presence and absence of hnRNP-K for 24 h. A cytoplasmic extract was prepared and centrifuged through a linear sucrose gradient to isolate polysomes. Total RNA was extracted from each of twelve sucrose gradient fractions. RT-PCR was performed using AR and β-tubulin primers. PCR products were separated on ethidium bromide stained 1% agarose gels. The polysome profile was determined by measurement of the OD260 for each fraction. **(D)** Polysome profile (OD260) and polysome analyses were performed in the presence of 200 μM puromycin. LNCaP cells were treated with indicated amount of puromycin for 30 min before sucrose gradient fractionation.

HnRNP-K inhibits PSA promoter activity and LNCaP cell proliferation. In order to determine whether AR down-regulation by hnRNP-K has functional consequences in PCa cells, we assessed the effect of enforced hnRNP-K expression on the androgen-responsive human prostate-specific antigen (PSA) promoter, and on LNCaP cell growth. Androgen-induced activation of the reporter gene was strongly and dose-dependently inhibited by hnRNP-K (Fig. 4A), indicating that hnRNP-K antagonizes androgenic signaling, consistent with a role as an AR antagonist. Similarly, hnRNP-K overexpression suppressed LNCaP cell proliferation (Fig. 4B), a result consistent with the PSA promoter-reporter data.

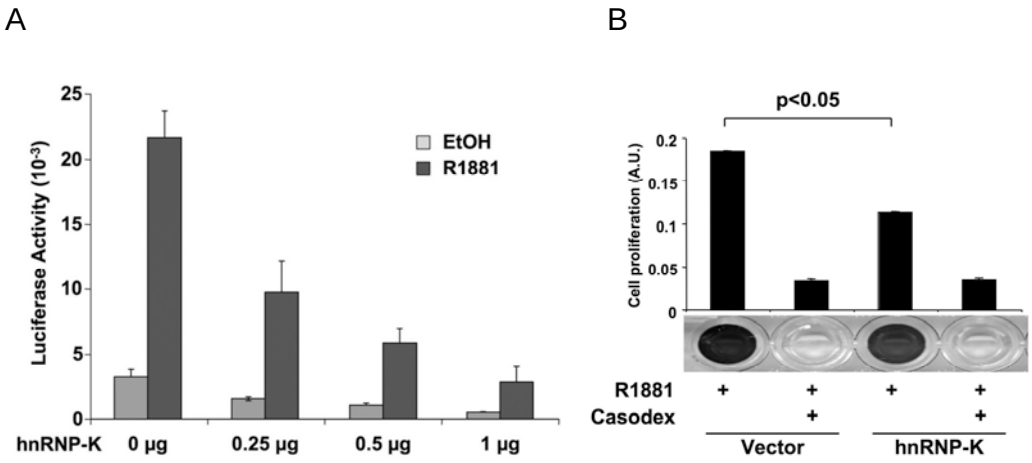

Figure 4. HnRNP-K inhibits AR-dependent gene expression and LNCaP cell growth. **(A)** Enforced expression of hnRNP-K dose dependently inhibits a luciferase reporter driven by the PSA promoter. **(B)** Enforced expression of hnRNP-K inhibits proliferation of androgen-dependent LNCaP cells. The AR inhibitor Casodex (bicalutamide) was used at a concentration of 10 µM.

HnRNP-K expression in human prostate cancer. Multiple data sets in the Oncomine cancer profiling database (www.oncomine.org) indicate that hnRNP-K is expressed in human PCa. We analyzed a human PCa TMA containing benign prostate tissues, organ-confined cancers, and metastases [hormone naive (HN) and hormone (castration) resistant (HR)] using a monospecific anti hnRNP-K antibody. HnRNP-K was detected in the nucleus of the majority of benign and carcinoma samples at high levels, and expression was significantly increased in metastatic tumors in comparison with benign tissues and organ-confined tumors ($p = 0.0001$ and 0.0004, respectively) (Fig. 5A, left panel), a trend that was maintained when HR metastases were compared with HN metastases (Fig. 5A, right panel). Notably, hnRNP-K and AR levels were significantly inversely correlated in localized PCa specimens ($p < 0.001$) (Fig. 5B). Finally, hnRNP-K was cytosolic and nuclear localized in organ-confined tumors, but cytosolic expression was significantly decreased in the metastases (Fig. 5C, D).

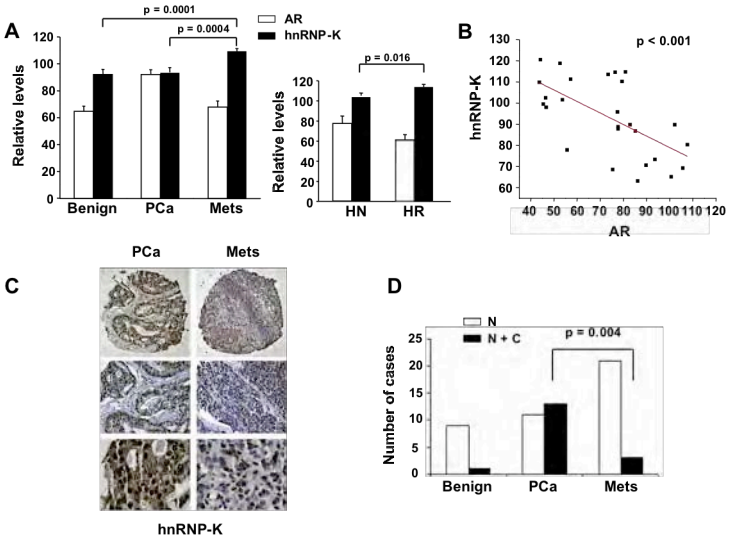

Figure 5. Quantitative analysis of hnRNP-K and AR levels in a human prostate TMA**. (A)** Relative levels of hnRNP-K and AR as assessed by ChromaVision analysis of TMA containing benign, local PCa, and metastatic tumors (left panel). The right panel shows averaged values of hnRNP-K and AR in the metastatic subset (HN: hormone naïve; HR: hormone resistant). **(B)** Regression analysis showing a significant inverse correlation between hnRNP-K and AR in organ-confined tumors. **(C)** Representative micrographs showing hnRNP-K expression and subcellular localization. Note the loss of cytoplasmic staining in

the metastatic tissue (lower right panel). **(D)** The graph shows the number of cases among the different diagnostic groups with predominant nuclear (N) or nuclear + cytosolic (N+C) staining. PCa, organ confined prostate cancer; Mets, metastases.

KEY RESEARCH ACCOMPLISHMENTS

- In year 1 we determined and reported the mechanism of regulation of AR expression by hnRNP-K
- Immunohistochemical analysis of a human PCa tissue microarray revealed an inverse correlation between hnRNP-K expression and AR protein levels in organ-confined prostate cancers and a substantial decline in cytoplasmic hnRNP-K in metastases, despite an overall increase in hnRNP-K levels in metastatic tumors. These findings are consistent with the possibility that the translational inhibitor mechanism we have identified is down-regulated with disease progression (i.e., cytoplasmic hnRNP-K is lost with progression).
- HnRNP-K is the first protein identified by any group that directly interacts with and regulates the AR translational apparatus.

REPORTABLE OUTCOMES

Mukhopadhyay, M., Kim, J., Cinar, B., Ramachandran, A., Hager, M.H., Di Vizio, D., Adam, R.M., Rubin, M.A., Raychaudhuri, P., De Benedetti, A., and Freeman, M.R**.** (2009) Heterogeneous nuclear ribonucleoprotein K is a novel regulator of androgen receptor translation. Cancer Res 69:2210-2218.

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CONCLUSIONS

This study has identified the RNA binding protein hnRNP-K as a physiologically relevant regulator of the androgen receptor in prostate cancer cells. Notably, hnRNP-K is the first protein ever shown to be a direct regulator of androgen receptor translation, despite reports from more than ***ten years ago*** that the AR can be regulated at the translational level. We are very encouraged by our data set at present as we move into year 2 of the project.

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